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Notch Regulation of Adam12 Expression in Glioblastoma Multiforme

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NOTCH REGULATION OF ADAM12 EXPRESSION IN GLIOBLASTOMA MULTIFORME

A Thesis Presented

by

ALA'A SALEM ALSYAIDEH

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DEDICATION

“Bear in mind that the wonderful things you learn in your schools are the work of many generations. All this is put in your hands as your inheritance in order that you may receive it, honor it, add to it, and one day faithfully hand it on to your children.“

- Albert Einstein

Dedicated to my loving husband, Basel Assaf, for his ever-lasting support and patience, and to my parents, Salem Alsyaideh and Fawziya Alshaar, for always pushing me to dream big.

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ABSTRACT

NOTCH REGULATION OF ADAM12 EXPRESSION IN GLIOBLASTOMA MULTIFORME

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Glioblastoma is the most common malignant brain tumor, accounting for 17% of all primary brain tumors in the United States. Despite the available surgical, radiation, and chemical therapeutic options, the invasive and infiltrative nature of the tumor render current treatment options minimally effective. Recent reports have identified multiple regulators of glioblastoma progression and invasiveness. It has been demonstrated that ADAM12, A Disintegrin And Metalloproteinase encoded by ADAM12 gene, is over-expressed in glioblastoma and directly correlated with tumor proliferation. Additionally, dysregulation of the Notch signaling pathway has been implicated in the pathogenesis of many gliomas. Lastly, an evolving role of microRNAs, small noncoding RNAs, in carcinogenesis is progressively growing. A recent study has identified ADAM12 as a notch-related gene, and another demonstrated that inhibition of notch signaling decreased glioblastoma recurrence. However the mechanisms of regulation are still unknown. In this study, we hypothesize that direct downregulation of microRNA-29, downstream of over-expression of notch, enhances glioblastoma malignancy through upregulation of ADAM12. Although our data demonstrate upregulation of Notch1, its downstream target

HES1, and ADAM12 in U87MG glioblastoma cell line. Expression of the cleaved intracellular Notch1 was not detected. Furthermore, we were unable to demonstrate an inhibitory effect of ɣ-secretase inhibitor on Notch signaling, likely reflecting the requirement for modifying culturing conditions or detection in our assays. Furthermore, miR-29 was detected in glioblastoma cells. The expression of miR-29 was further elevated by ɣ-secretase inhibitor treatment, suggesting a role for Notch1 inhibition on miR-29 expression. Although no conclusive results are shown in our work, a role of Notch1 through miR-29 is implicated in the pathogenesis of glioblastoma pathogenesis warranting further investigation into the role downstream target genes in the Notch signaling pathway.

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CHAPTER 1

THE ROLE OF NOTCH SIGNALING IN THE PATHOGENESIS OF GLIOBLASTOMA MULTIFORME

1.1. Glioblastoma

1.1.1. Introduction

Gliomas are heterogeneous brain tumors with cellular features of glial cells and include astrocytomas, oligodendrogliomas, ependymoma, and mixed gliomas. Among the most aggressive subtypes is Glioblastoma Multiforme (GBM), the most aggressive of astrocytomas classified as Grade IV astrocytic neoplasm according to World Health Organization (WHO) (Louis et al., 2007). Histologically, GBM is characterized by increased cellularity, nuclear atypia, and mitotic activity similar to Grade III Anaplastic Astrocytoma, in addition to microvascular proliferation and necrosis unique to GBM (Figure 1) (Wen and Kesari, 2008). GBM is the most common primary brain neoplasm usually found in the cerebral hemispheres, but may also occur in the cerebellum, brainstem, or spinal cord (Schwartzbaum et al., 2006). Depending on the involved brain site, the most common clinical signs include seizures, headaches, and focal neurologic deficits. GBM accounts for 80% of all malignant brain tumors (CBTRUS, 2010). The causes of GBM are currently unknown; however, multiple risk factors, molecular pathways, and potential etiologies have been implicated in cellular transformations and tumorigenesis. Alcohol consumption, ionizing radiation, Simian virus 40 (SV40), and cytomegalovirus are among the most recent factors implicated in GBM pathogenesis (Baglietto et al., 2011; Cavenee, 2000; Dziurzynski et al., 2012; Vilchez et al., 2003).

Due to its infiltrative and necrotic potentials in addition to the uncontrolled cellular proliferation, angiogenesis, and resistance to apoptosis, GBM is associated with very poor prognosis (Furnari et al., 2007), with survival rates of only 4.5% or less (CBTRUS, 2010). Although combination of surgical, radiotherapy, and chemotherapy exists, the infiltrative nature of GBM leads to incomplete recovery and frequent fatal tumor relapse. This is primarily due to the difficulty in overcoming resistant cancer stem cells (CSCs).

1.1.2. Cellular Origin

GBM is composed of a mixed population of cell types that includes astrocyte-like and stem-like cells. The cellular origin of GBM is a controversial topic in glioma cancer research, and a continuous interest in GBM ontogeny is stimulated for the possibility of better understanding of fundamental pathways necessary for GBM development that could aid in improving diagnostic and therapeutic solutions (Visvader, 2011). One of the most significant recent advances in this regard is the discovery of cancer stem cells (CSC). For those pertaining to GBM, CSCs share multiple features with those found in normal neural stem cells and progenitor cells, such as self-renewal, marker expression, multilineage differentiation, and localization to stem cell microenvironment niches (Sanai et al., 2005). More recently, by introduction of genetic mutations into neural stem cells, several studies have supported the notion of neural stem cells in the production of GBM (Bachoo et al., 2002; Zheng et al., 2008; Zhu et al., 2005). Moreover, neural stem cells are probably target cells for malignant transformation, and the wide range in GBM heterogeneity represents different stages of neural stem cell differentiation hierarchy. Regardless, common features need to be met for a cell to be considered glioma CSC (Rich, 2008), which include capability of self-renewal, high proliferative potential, and when injected in xenograft models to initiate tumorigenesis. Despite the scientific

advances, it's currently debatable whether CSCs originate from neural stem cells. CSCs are identified immunocytochemically by expression of CD133 (Assanah et al., 2006; Beier et al., 2007; Kesari and Stiles, 2006; Singh et al., 2004), and constitute a minority of the GBM cell population. Despite that, they play a critical role in tumor generation and maintenance, and contribute to tumor resistance to standard treatments (Dirks, 2008; Vescovi et al., 2006).

1.1.3. Molecular Pathogenesis

The mechanism of glial transformation is the result of cumulative genetic mutations and growth-factor signaling pathway dysregulation. Dysregulated signaling pathways frequently result in transcriptional activation of survival, proliferation, invasion, and angiogenesis pathways (Figure 2).

1.1.3.1. Growth Factor Overexpression and Apoptosis

Expression of both growth factors and their receptors is common in GBM and sets up an autocrine growth-promoting loop. The most common signaling defects occur in those that involve Epidermal Growth Factor Receptor (EGFR) and Platelet-derived Growth Factor Receptor (PDGFR) (Furnari et al., 2007). The A chain of PDGF and its cognate alpha receptor are overexpressed in the majority of diffuse astrocytic tumors (Hermanson et al., 1992). Amplification of EGFR expression on the other hand occurs primarily in GBM (Wen and Kesari, 2008). Upregulation of EGFR is reported in 40-50% of GBM patients, and half of those express a constitutively autophosphorylated EGFRvIII variant (Furnari et al., 2007; Pelloski et al., 2007). Aberrant growth factors result in activation of intermediate signal-transduction pathways; most commonly the Ras–mitogen-activated protein (MAP) kinase pathway involved in proliferation and cell cycle progression, the phosphatidylinositol 3-kinase (PI3K)–Akt–mammalian target of

rapamycin (mTOR) pathways leading to the inhibition of apoptosis and enhancing cellular proliferation, and the inactivation of tumor-suppressor gene PTEN which negatively regulates the PI3K pathway (Furnari et al., 2007).

1.1.3.2. Aberrant Cell Cycle

Like other cancers, perturbations of p53- and Rb-mediated cell cycle regulation occur commonly in GBM. p53 protein normally inhibits cell cycle progression and induces apoptosis in response to DNA damage, and hence inactivation of p53 function leads to cell cycle dysregulation. Mutation in p53 is reported in approximately 25% of GBMs (Fulci et al., 1998). Aberrant loss of Rb and other components of the p16/cdk4/cyclinD/pRb G1 to S phase transition cell cycle regulation are frequently observed in GBM and other astrocytic tumors. Mutation in Rb occurs in 30–40% of GBMs, and mutation in p16 occurs in 33% of GBM. Disturbance of this check point results in uncontrolled cell cycle and progression of tumor formation.

1.1.3.3. Angiogenesis and Invasion

GBM is a markedly vascularised cancer and characterized by significant invasiveness of the surrounding brain parenchyma. This is heavily regulated through crosstalk between growth factors, extracellular matrix proteins, integrins, proteinases, and tissue microenvironment. Angiogenic and invasive features of GBM result from a combination of stimulating factors, such as activation of vascular endothelial growth factor (VEGF) and Matrix Metalloproteinases (MMPs) (Guo et al., 2003; Jain et al., 2007), that aid in neovascularization and breakdown of impeding extracellular matrix proteins, and degradation of normally inhibiting factors, such collagen, leading to local tumor spread and access to the systemic circulatory system.

1.1.3.4. Other signaling pathway

Other developmental pathways that contribute to the biologic features of gliomas are those involving Notch, sonic hedgehog, wingless, CXC chemokine receptor 4 (CXCR4), and bone morphogenetic proteins (Lee da and Gutmann, 2007). In the following section, we will review details on Notch biology and its contribution to GBM tumorigenesis.

1.2. Notch

1.2.1. Biology

The "Notch" family of proteins was initially identified by Thomas Morgan in *Drosophila melanogaster* in 1917. Mutation in the genes encoding Notch proteins resulted in 'notches' at the wing margin of adult flies, hence inspiring the Notch name (Morgan, 1917). Notch proteins are type I transmembrane receptors consisting of 36 ligand binding epidermal growth factor (EGF)-like tandem repeats and 3 LIN-12/Notch (LIN) repeats in the extracellular domain. The intracytoplasmic domain contains RBPJκ-binding (RAM) domain, six tandem ankyrin (ANK) repeats, a transcription activation domain (TAD) and a proline/glutamate/serine/threonine-rich (PEST) sequence (Figure 3) (Tsukumo and Yasutomo, 2004). Notch receptor is cleaved post-translationally at site S1 and heterodimerized between two extracytoplasmic juxtamembrane regions that form both N-terminal and C-terminal heterodimerization domains (HD-N and HD-C, respectively) to generate a fully mature receptor. In vertebrates, four Notch paralogs are encoded (Notch 1-4), with Notch1 and Notch2 showing high homology with *Drosophila* Notch, and Notch3 and Notch4 are more distantly related, with 34 and 29 EGF-like repeats, and are devoid of TAD domains (Lardelli et al., 1994; Uyttendaele et al., 1996; Weinmaster et al., 1991; Weinmaster et al., 1992).

1.2.2. Notch signaling

In mammals, Notch receptors are activated by five type I transmembrane ligands, three Delta-like (Dll1, Dll3 and Dll4) and two Serrate/Jagged (Jag 1 and Jag2) (Figure 3) (Artavanis-Tsakonas et al., 1999).

Notch activation results from ligand-mediated cleavage at sites S2 or S3 (Figure 4). Activation of Notch from cleavage at site S3 within the transmembrane domain is mediated by gamma-secretase (De Strooper et al., 1999). Receptor activation leads to release of Notch intracellular domain (NICD), which translocate to the nucleus. Thereafter, intranuclear NICD binds Su(H)/CSL/CBF1/RBPJκ to trans-activate target genes such as HES and HEY families of basic helix-loop-helix transcription factors (Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999; Jarriault et al., 1995). Cleavage of Notch at site S2 is mediated by an ADAM metalloprotease and TNF-α-converting enzyme (TACE) (Brou et al., 2000), and leads to the release of a soluble form of Notch named Notch extracellular truncation (NEXT) (Mumm et al., 2000).

Non-canonical Notch signaling can occur independent of RBPJκ, presumably through a Deltex-dependent alternative pathway (Matsuno et al., 1998; Ramain et al., 2001). In T helper (Th) cells, Jagged mediates Th2 cell differentiation by triggering the RBPJκ-dependent canonical pathway, while Dll1 instructs Th1 commitment in an RBPJκ-independent fashion (Amsen et al., 2004).

1.2.3. Notch in Brain Development

The Notch signaling pathway plays a fundamental role in the regulation of several developmental processes such as proliferation, stem cell maintenance, differentiation during embryonic development and homeostasis of adult self-renewing organs (Bray, 2006; Wen and Kesari, 2008). Canonical and non-canonical Notch signaling pathways have been shown to target several genes involved in brain development. The canonical pathway has been shown to target GFAP (Ge et al., 2002), HES1 (Wu et al., 2003), HEY1 (Zavadil et al., 2004), BLBP (Patten et al., 2003), NESTIN (Shih and Holland, 2006), and TNC genes (Sivasankaran et al., 2009), while the non-canonical pathway has been shown to target ERBB2 (Patten et al., 2006). Most importantly, Notch1 induces TP53-dependent EGFR expression (Purow et al., 2008), the most common signaling defect in GBM.

In addition to the "notched" winged observed in *Drosophila melanogaster* with mutated Notch genes, several studies have demonstrated the role of Notch in neurogenesis. In loss-of-function experiments, a defective neuroectodermal development was observed, with the development of an embryonic phenotype characterized by expanded population of neuroblasts at the expense of epidermal cells (Poulson, 1937). This indicated that Notch is an antineurogenic factor required to control cell fate decision by preventing the differentiation into neuroblasts. Gain-of-function mutation in Notch gene further supported the antineurogenic role of Notch during *Drosophila* development (Brennan et al., 1997; Rebay et al., 1993).

Distinct patterns of Notch expression are seen in rodents. While *notch1*, *notch2*, and *notch3* transcripts are commonly present in the germinal zones of late embryonic and postnatal brains, *notch2* expression persists in the postnatal mouse brain in immature glial

cells (Higuchi et al., 1995; Irvin et al., 2001; Tanaka et al., 1999). Consistent with the antineurogenic role of Notch signaling in *Drosophila,* it appears that high expression of Notch2 is required for the prevention of neuronal differentiation and the maintenance of neural stem cell (NSC). Activation of Notch promoted gliogenesis at the expense of neurogenesis (Morrison et al., 2000), and suppressed neuronal differentiation of NSCs by maintaining their proliferation (Hitoshi et al., 2002; Shimizu et al., 2008; Solecki et al., 2001). These observations suggest a role for Notch signaling in the maintenance of GBM CSC.

While knockout of Notch1 and Notch2 in results in embryonic and perinatal lethality, mutations in Notch have been associated with neurological and vascular abnormalities in humans (Garg et al., 2005; Joutel et al., 1996). The fact that the Notch signaling pathway is involved in several developmental processes and that inactivation of Notch results in constant defects in angiogenesis implicate a possible role for Notch signaling in tumorigenesis and tumor neovascularization. This is evident in the role of Notch1 and Notch2 mutations found in acute T cell lymphoblastic leukemia (T-ALL) and a fraction of B cell lymphomas, respectively (Ellisen et al., 1991; Lee et al., 2009).

1.2.4. Notch in Glioblastoma

Notch signaling is one of the major pathways involved in GBM development, and expression of Notch proteins and their ligands are critical markers of GBM survival and proliferation (Boulay et al., 2007; Purow et al., 2005).

Notch1 and Notch2 are highly expressed in GBM and astrocytoma (Sivasankaran et al., 2009). Notch2 has been suggested to drive embryonic brain tumor growth (Solecki et al., 2001), with high expression in GBM (Sivasankaran et al., 2009). Through TP53, Notch1 was shown to regulate transcription of the EGFR gene, the major proliferation

pathway in GBM (Purow et al., 2008), with overexpression of Notch signaling mediator genes in the molecular subset of GBM with EGFR amplification (Brennan et al., 2009). In contrast, a minor subset of GBM with slower GBM progression and better patient prognosis are associated with impaired Notch2 signaling and impaired Notch-mediated GBM invasiveness (Boulay et al., 2007; Sivasankaran et al., 2009). The role of Notch was further supported by loss-of-function and gain-of-function experiments. While inhibition of Notch signaling by the use of ɣ-secretase inhibitors (GSIs) resulted in reduction in GBM CSC proliferation and increased apoptosis, while expression of an active form of Notch2 increased tumor growth (Fan et al., 2009). Interestingly, *in vivo* delivery of GSI consistently blocked tumor growth, and significantly prolonged patient survival (Fan et al., 2009). Through the inhibition of Notch signaling, GSI treatment significantly reduced radioresistance of GBM CSC (Wang et al., 2009).

Additionally, Notch-RBPJκ transactivates tenascin-C (Sivasankaran et al., 2009), a highly expressed extracellular matrix glycoprotein that increases during progression of GBM and used as a prognostic marker for GBM patient survival (Garcion et al., 2001; Leins et al., 2003).

1.3. MicroRNAs

1.3.1. Signaling and processing of miRNAs

MicroRNAs (miRNAs) are a non-coding class of RNAs, 20-25 nucleotides in length that do not result in proteins. MicroRNAs modulate gene expression through base pairing between the seed sequence of the miRNA (nucleotides 2-8 at its 5' end) and their complementary match sequence at the 3' UTR region of target mRNAs (Bartel, 2009). Cognate binding of miRNA to its target mRNA results in translation suppression or direct degradation of targeted mRNA (Kolfschoten et al., 2009). MicroRNAs are first

transcribed as long (~ 250 nts) primary transcripts (pri-miRNA) that fold into hairpin-like structures. Following transcription, primary transcripts are processed in the nucleus by the Drosha complex into pre-miRNAs (~120 nts) and transported to the cytoplasm by exportin 5. In the cytoplasm, pre-miRNAare further processed by Dicer ribonuclease (Carthew and Sontheimer, 2009), in which mature miRNAs are incorporated into the RNA-induced silencing complex (RISC) in association with Argonaute proteins. Single-stranded miRNA is unwound by Dicer, which also guides target selection, inhibiting the translation, and stability and/or localization of target mRNA (Brodersen and Voinnet, 2009; Kim et al., 2009; Zheng et al., 2008). MicroRNA biogenesis and mRNA binding is illustrated in figure 5.

1.3.2. Structure and function of the miR-29 family

The microRNA-29 (miR-29) family in human consists of four members: miR-29a, miR-29b1, miR-29b2, and miR-29c. They are encoded by two gene clusters, MIR-29B1-MIR-29A and MIR-29B2-MIR-29C located in chromosome 7 (chr.7q32.3) and chromosome 1 (chr.1q32.2), respectively (Kriegel et al., 2012). miR-29b1 and miR-29b2 are identical, but they are distinguished from each other due to their chromosomal location. The distance between the two miRNAs in each cluster is less than 1kb (Chang et al., 2008; Eyholzer et al., 2010; Mott et al., 2010), and miR-29b1 and miR-29b2 are upstream of miR-29a and miR-29c, respectively the gene sequences are highly conserved between humans, rats and mice. The mature sequences of miR-29a and miR-29c are 22 nucleotides long while miR-29b1/b2 have identical mature sequences and are 23 nucleotides long. Each of miR-29 members have the same short seed sequence; AGCACC between positions 2 to 7 in each mature sequence. Consequently, it is

predicted that all miR-29 members target the same set of genes. The structures of pre-miR-29 and mature miR-29 are shown in Figure 6.

1.3.3. The miR-29 family in tumorigenesis

Similar to mRNAs encoding for proteins, miRNAs have well-defined developmental and cell-type-specific expression patterns, and may be involved in tumor suppression or tumor malignancies (Havelange et al., 2009).

Loss of miR-29 function has been associated with several tumors. Mutated miR-29b has been shown in leukemia, and mutation repair prevented the ability of leukemia cells from growing in an immunodeficient mouse host (Havelange et al., 2009). Upregulated miR-29a and miR-29b were also found in B-cell Chronic Lymphocytic Leukemia (CLL) (Santanam et al., 2010). Elevated miR-29a expression and downregulation of miR-29b were found in primary Acute Myeloid Leukemia (AML) (Garzon et al., 2008; Han et al., 2010). Reduced expression of miR-29b has been observed in hepatocellular carcinoma and is significantly associated with poor prognosis. Re-introduction of miR-29a/b/c induced apoptosis of cancer cells and repressed tumorigenicity (Xiong et al., 2010). miR-29b was similarly found downregulated in cholangiocarcinoma cell lines (Mott et al., 2007). Furthermore, miR-29 was found to function in myogenesis and is epigenetically silenced in rhabdomyosarcoma cell lines (Wang et al., 2008). Upregulation of miR-29a/b/c expression was also found in pancreatic endocrine tumors (Roldo et al., 2006).

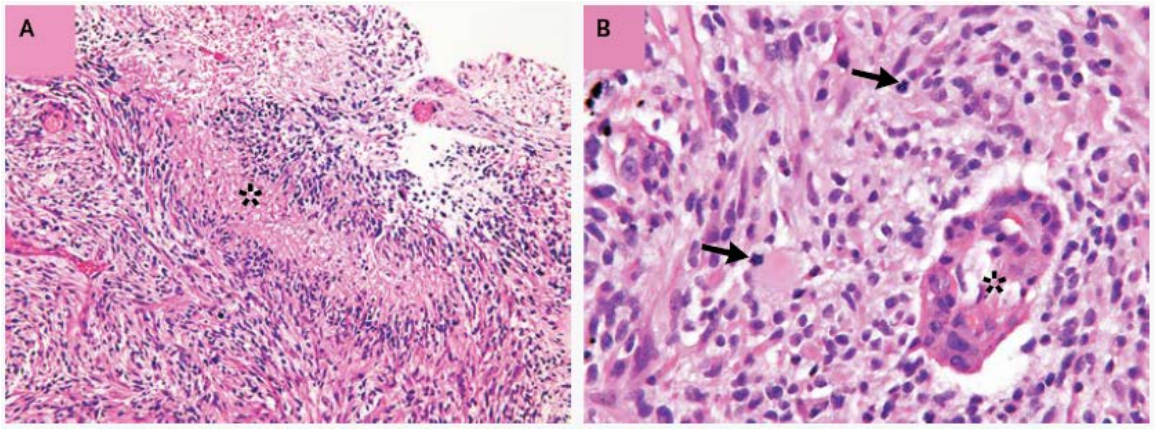

Figure 1: Histological features of Glioblastoma Multiforme. Panels A and B show the histologic appearance of a glioblastoma, characterized by nuclear pleomorphism, dense cellularity, and pseudopalisading necrosis (asterisk) (Panel A, hematoxylin and eosin), as well as vascular endothelial proliferation (asterisk) and mitotic figures (arrows) (Panel B, hematoxylin and eosin) (Wen and Kesari, 2008).

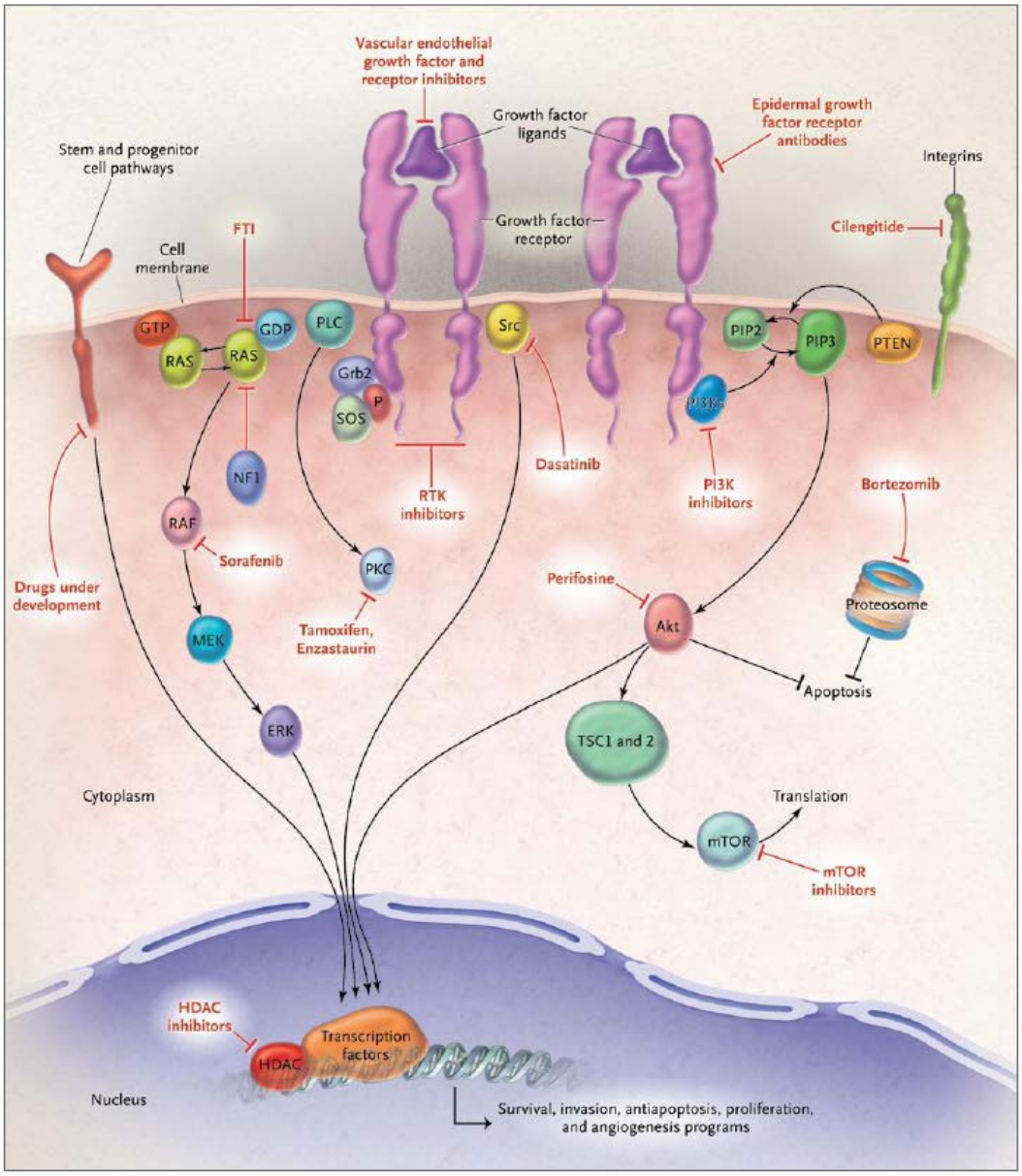

Figure 2: Major Signaling Pathways in Malignant Gliomas and the Corresponding Targeted Agents in Development for Glioblastoma (Wen and Kesari, 2008).

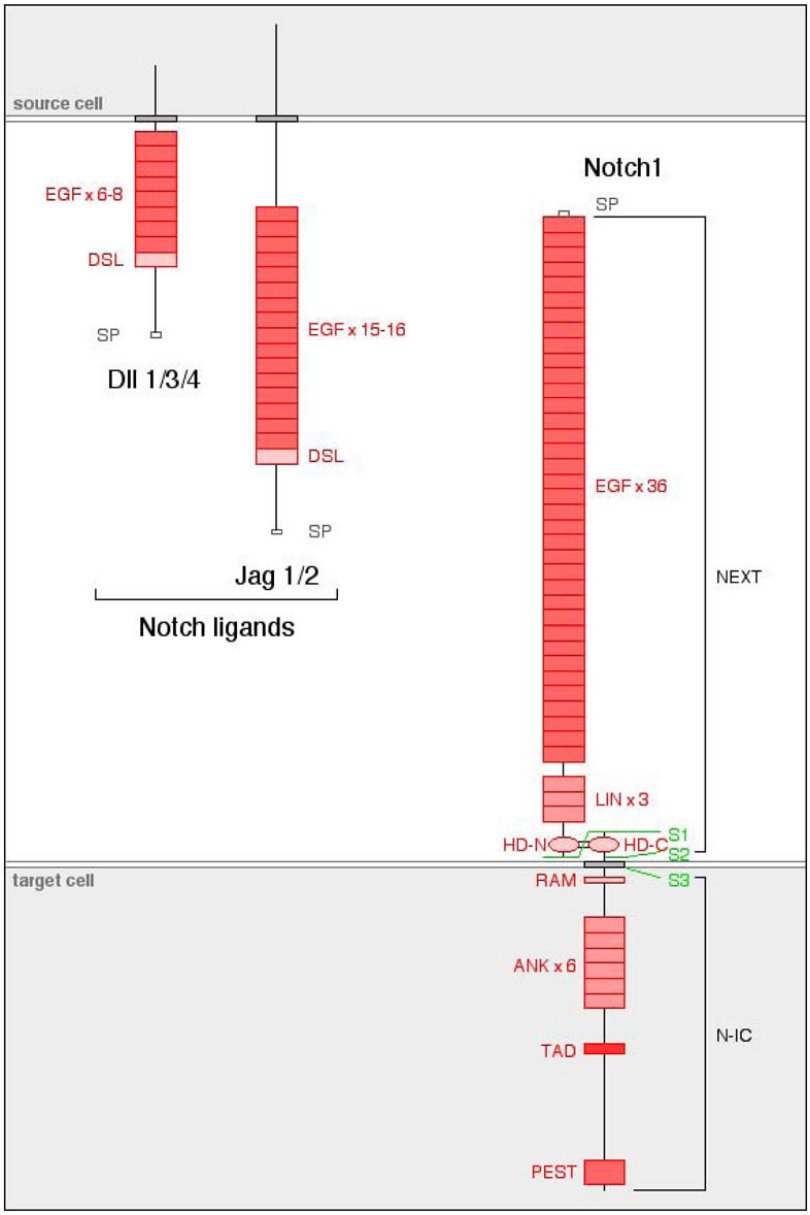

Figure 3: Ligands, structure and processing of Notch1 receptor. Left: the Delta-like ligand (Dll) and Serrate-Jagged ligand (Jag) structural subsets of Notch ligands. Right: structure of Notch1 receptor resulting from post-translational cleavage at site S1 and heterodimerization of the cleaved parts. Ligand-dependent cleavages at sites S2 and S3 generate soluble Notch extracellular truncation (NEXT) and cytosolic Notch intracellular domain (N-IC) forms, respectively (Lino et al., 2010).

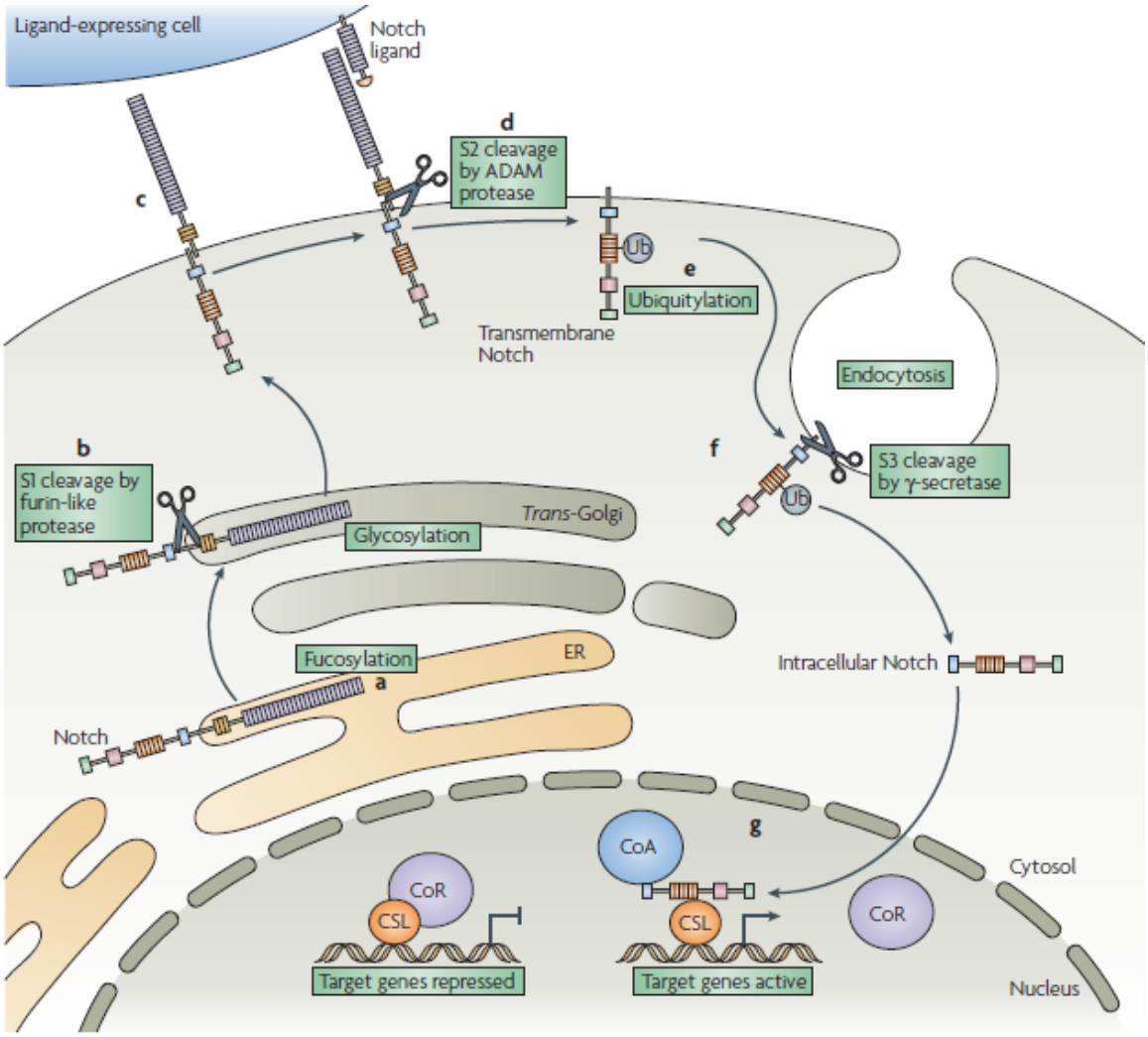

Figure 4: Sequential events and control of Notch expression and signaling (Osborne and Minter, 2007)**.**

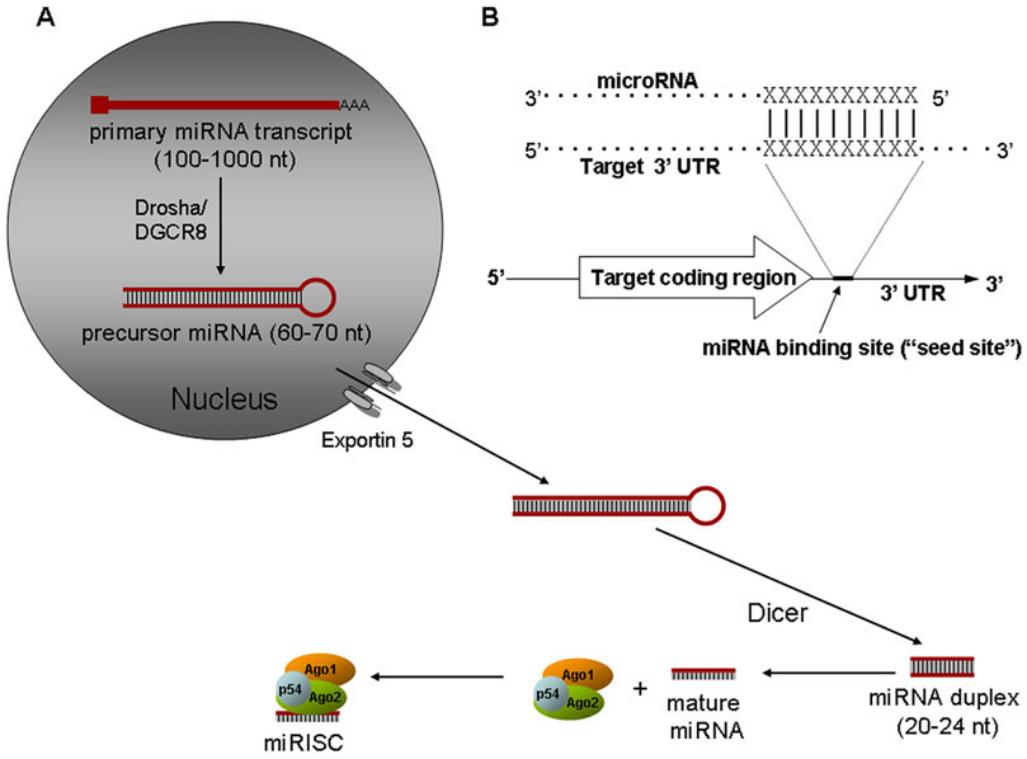

Figure 5.Schematic of microRNA biogenesis. (A) Biogenesis of a miRNA-induced silencing complex (RISC). (B) Binding of microRNA to the 3'-UTR of mRNA (Liu, 2009).

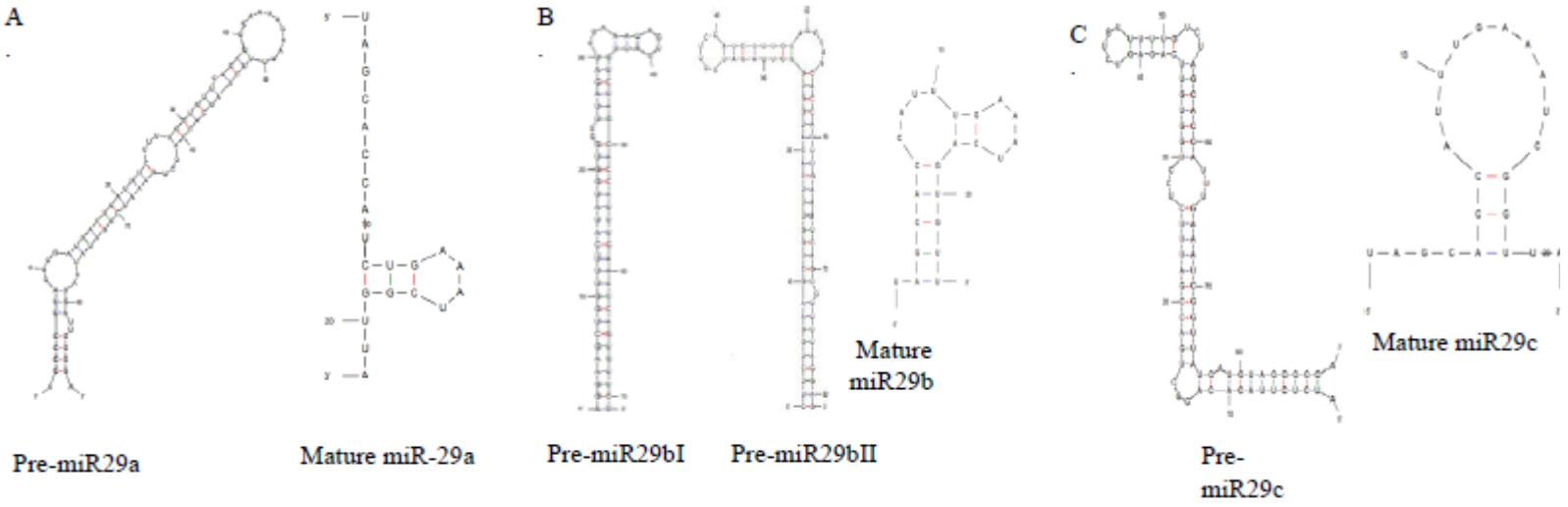

Figure 6: Structure of pre-miR-29 and mature miR-29 family of microRNAs. The structure of (A) miR-29a, (B) miR-29b and (C) miR-29c.

1.4. AIM of the study

1.4.1. Rationale

Recent reports have identified multiple regulators of glioblastoma progression and invasiveness. It has been demonstrated that ADAM12, A Disintegrin and Metalloproteinase encoded by *ADAM12* gene, is over-expressed in glioblastoma and directly correlated with tumor proliferation. Additionally, dysregulation of the Notch signaling pathway has been implicated in the pathogenesis of many gliomas. Lastly, an evolving role of microRNAs in carcinogenesis is progressively growing. A recent study has identified *ADAM12* as a Notch-related gene, and another demonstrated that inhibition of Notch signaling decreased glioblastoma recurrence. However the mechanisms of regulation are still unknown.

1.4.2. Hypothesis

In this study, we hypothesize that direct downregulation of microRNA-29, downstream of over-expression of Notch, enhances glioblastoma malignancy through upregulation of ADAM12.

CHAPTER 2

RESULTS

2.1. Assay optimization for the detection of Notch1 in GBM cell line

For optimal detection of Notch1 and ADAM12 in GBM cell line, U87MG cells were plated at different cellular density at two culture durations. Cells were cultured at $0.25X10^6$, $0.5X10^6$, and $1X10^6$ cells per well in 6-well plates and were incubated at 37˚C/5%CO_2 for 24 hours and 48 hours. Cells were harvested and examined by trypan blue viability assay as indicated in the Materials and Methods section.

As shown in Table 1, all culture conditions resulted in an increase in the total number of cells after 24 and 48 hours of culture initiation. While the percent viability was high in all conditions, initial culture start of $0.25X10^6$ cells for 24 hours results in the most remarkable cell death and lowest viability percentage at 57.1% despite the increase in total number of cells 24 hours post culture initiation. Viability percentage of initial $0.25X10^6$ cells condition was higher at 48 hours post culture initiation. Culturing cells at $0.5X10^6$ and $1X10^6$ resulted in cellular expansion and comparable high viability rates at both 24 and 48 hours.

2.2. Expression of Notch1, ADAM12, and miR-29 in GBM cell line

The Notch signaling pathway has been implicated in the pathogenesis of GBM. To examine the expression of Notch signaling pathway components in U87MG GBM cell line, Notch1, HES1, and c-myc expression levels were determined at the protein and mRNA levels. Additionally, ADAM12 expression levels were determined to correlate

their expression levels with components of the Notch signaling pathway. Cell culture conditions of $0.3X10^6$ per well were plated in a 6-well plate and incubated for 24, 48, and 72 hours to monitor the differential Notch1 and ADAM12 protein expressions at different time points (Figure 7). Notch1-specific antibody used in these verification experiments is able to detect the C-terminus of Notch1 present in uncleaved Notch1 and the transmembrane domain post Notch1 activation. Our ADAM12-specific antibody is able to detect both the long and short forms of ADAM12. Jurkat cells were used as a positive control for the detection of Notch1 and ADAM12, and DO11.10 were used as a negative control for Notch1 expression.

Both total Notch1 (300kDa, not shown) and the cleaved transmembrane domain of Notch1 (120kDa) were detected at all time points in both the GBM U87MG cell line and in the positive control Jurkat cell line. Two bands were present in the negative control DO11.10 cell line; however, these bands don't correspond to the expected band sizes of Notch1 and hence likely represent non-specific cross reactivity. The downstream components of the Notch signaling pathway, HES1 and c-myc, were detected in U87MG cell line by western blot analysis (data not shown). ADAM12 was equally detected at all time points, correlating with the upregulated expression of Notch signaling pathway components. Adequate protein extraction and equal protein loading per lane were confirmed by the strong and equal band density of the positive internal control β-Actin.

To verify the activity of the Notch signaling pathway at the transcriptional levels, mRNA expression of *NOTCH1*, *HES1*, and the long (*ADAM12L*) and short (*ADAM12S*) forms of ADAM12 were measured using the same culture conditions (Figure 8). *NOTCH1*, *HES1*, *ADAM12L*, and *ADAM12S* were detected to levels comparable to the

positive internal control *β-ACTIN*, indicating the activation of these genes in U87MG GBM cell line. Transcript levels of all genes remained relatively constant at 24, 48, and 72 hours post culture initiation. *ADAM12L* expression was higher compared to *ADAM12S* and *NOTCH1*, the latter of which had comparable expression levels.

We further demonstrated the specificity and sensitivity of our culture conditions and assay design for the detection of members of the miR-29 family, miR-29a, miR-29b, and miR-29c, a group of microRNAs that are modulated by Notch1 activation in certain types of tumors (Figure 9). Sno202 was used as an internal control, and it was detected in all culture conditions. All members of the miR-29 family were detected in our assay to levels below sno202. When compared to each other, levels of miR-29a, miR-29b, and miR-29c were comparable at all time points. In general, the expression levels of all miR-29 members were approximately 50% of the expression level of the internal control sno202.

Repetition of the same quantitative RT-PCR work with lower cell density per well at $0.2X10^6$ cells per well in 6-well plate resulted in similar expression levels of *NOTCH1*, *HES1*, *ADAM12L*, and *ADAM12S* (Figure 10). Although miR-29 expression was detected at lower cell culture density, the expression levels were nearly 1.3 fold higher compared to their corresponding expression levels at $0.3X10^6$ cells per well. Despite lower viability rates at lower cell density, expression levels remained comparable between the two culture conditions. Regardless, our results demonstrate that our culture conditions and detection assays are sufficient and capable of detecting variable protein and transcript levels of Notch1, HES1, ADAM12, and members of miR-29 family.

2.3. The effect of gamma-secretase inhibitor on the expression of Notch1, HES1, ADAM12, and miR-29 in GBM cell line

To explore the regulation of Notch signaling pathway in GBM, we treated U87MG GBM cell line with ɣ-secretase inhibitor (GSI), an inhibitor of Notch cleaving enzyme ɣ-secretase, and measured gene transcription levels of components of the Notch signaling pathway. Using western blot analysis, full-length Notch1 and the cleaved transmembrane component of Notch1 were readily expressed in U87MG cells (Figure 11). The cleaved intracellular component of Notch1 however was not detected. Similarly, ADAM12 was detected in cells. Treatment of cells with GSI did not affect the protein expression levels of Notch1 or ADAM12. Repeating the same experiment at longer durations of 48h and 72h with longer and higher concentrations of GSI treatment did not result in any difference in the Notch1 and ADAM12 expression levels, and the cleaved intracellular domain remained undetectable.

To verify the effectiveness of GSI treatment, similar conditions were carried out on Jurkat cell line, cells known to constitutively express high levels of the cleaved intracellular component of Notch1 and respond to GSI treatment (Figure 12A). Indeed, the cleaved intracellular component of Notch1 was detected in untreated Jurkat cells. Moreover, treatment of cells with multiple preparations of GSI resulted in significant downregulation of the cleaved intracellular component of Notch1 as expected. These results demonstrate that our culture setup and treatment are functioning properly. We then tested the same GSI preparations on U87MG GBM cell line (Figure 12B). While the Notch1 and ADAM12 were expressed, the cleaved intracellular component of Notch1 was not expressed. Furthermore, treatment of cells with different GSI preparations for 48h did not result in changes in the levels of Notch1 or ADAM12 expression. These

results demonstrate that Notch1 has been cleaved, as detected by the cleaved transmembrane component, the cleavage of Notch1 did not result in an intracellular component, suggesting one of the following scenarios: (1) that cleaved intracellular component of Notch1 rapidly degraded to levels below our assay limits of detection in whole cell lysate and that Notch1 enrichment in nuclear protein fraction is necessary for the detection of low levels of cleaved intracellular Notch1, or that (2) Notch1 was cleaved at a site alternative to V1744, rendering our detecting monoclonal antibody non-specific for the detection of newly formed cleaved Notch1 in our western blot analysis.

To further investigate the expression of the Notch signaling pathway at the transcriptional levels using our quantitative RT-PCR assay. Relative to *β-ACTIN* expression, the expression of *NOTCH1* transcript levels increased with GSI treatment (Figure 13A). *NOTCH1* expression levels were highest at 72h post treatment, with expression levels at least 4 fold higher compared to *β-ACTIN* expression and 10 fold higher with 50μM GSI treatment. The expression of *HES1*, *ADAM12L*, and *ADAM12S* were similar to those seen with *NOTCH1*. *HES1* expression was 3-20 fold higher than *β-ACTIN* expression, particularly with 5μM and 50μM GSI treatment at 24h and 72h post treatment (Figure 13B). Levels of *ADAM12L* were at least 2 fold higher than *β-ACTIN* expression; however, this was only seen with 72h of culture treatment (Figure 13C). *ADAM12S* was similarly expressed at high levels, particularly with 5μM and 50μM GSI treatment at all time points (Figure13D). These results demonstrate difference between the transcriptional and protein levels of the examined genes, and indicate that the mode of regulation in U87MG GBM cell line needs to be explored.

In an attempt to investigate the regulation of the aforementioned genes, we measured the expression levels for members of the miR-29 family. Levels of miR-29a, miR-29b, and miR-29c were constantly elevated compared to the internal control (Figure 14). Levels of miR-29b and miR-29c at 72h post treatment were the highest. Although further work is needed to investigate the nature of miR-29 involvement in GBM pathogenesis, the elevated levels of miR-29 members support their role in GBM pathogenesis.

Table 1. Viability Assay of U87MG cell line at 24 and 48 hour.

24 HOUR CULTURE					
ORIGINAL NUMBER	**TOTAL LIVE**	**TOTAL DEAD**	**TOTAL ALL**	**% VIABLE**	**% DEAD**
$0.25X10^6$	$2.0X10^5$	$1.5X10^5$	$3.5X10^5$	57.1	42.9
$0.5X10^6$	$5.5X10^5$	$1.0X10^5$	$6.5X10^5$	84.6	15.4
$1.0X10^6$	$1.1X10^6$	$7.5X10^4$	$1.1X10^6$	93.3	6.7
48 HOUR CULTURE					
ORIGINAL NUMBER	**TOTAL LIVE**	**TOTAL DEAD**	**TOTAL ALL**	**% VIABLE**	**% DEAD**
$0.25X10^6$	$3.5X10^5$	$1.0X10^5$	$4.5X10^5$	77.8	22.2
$0.5X10^6$	$5.8X10^5$	$5.0X10^4$	$6.3X10^5$	92.0	8.0
$1.0X10^6$	$1.2X10^6$	$2.5X10^5$	$1.5X10^6$	83.1	16.9

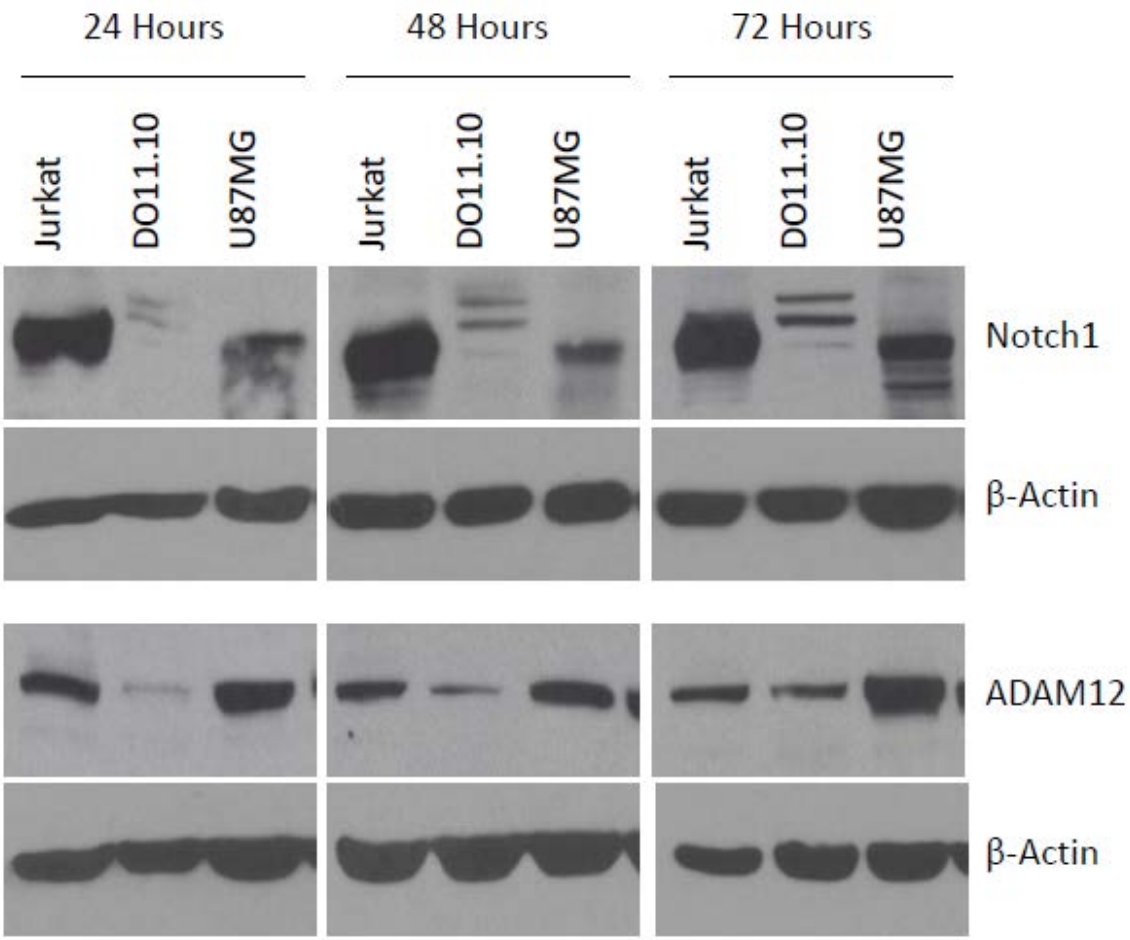

Figure 7: Expression of Notch1 and ADAM12 in U87MG GBM cell line. Western blot analysis of protein levels of Notch1 and ADAM12 in U87MG GBM cell line at 24h, 48h, and 72h of culture. Jurkat cells were used as a positive control for the expression of Notch1 and ADAM12 and DO11.10 cells were used as a negative control for the expression of Notch1.

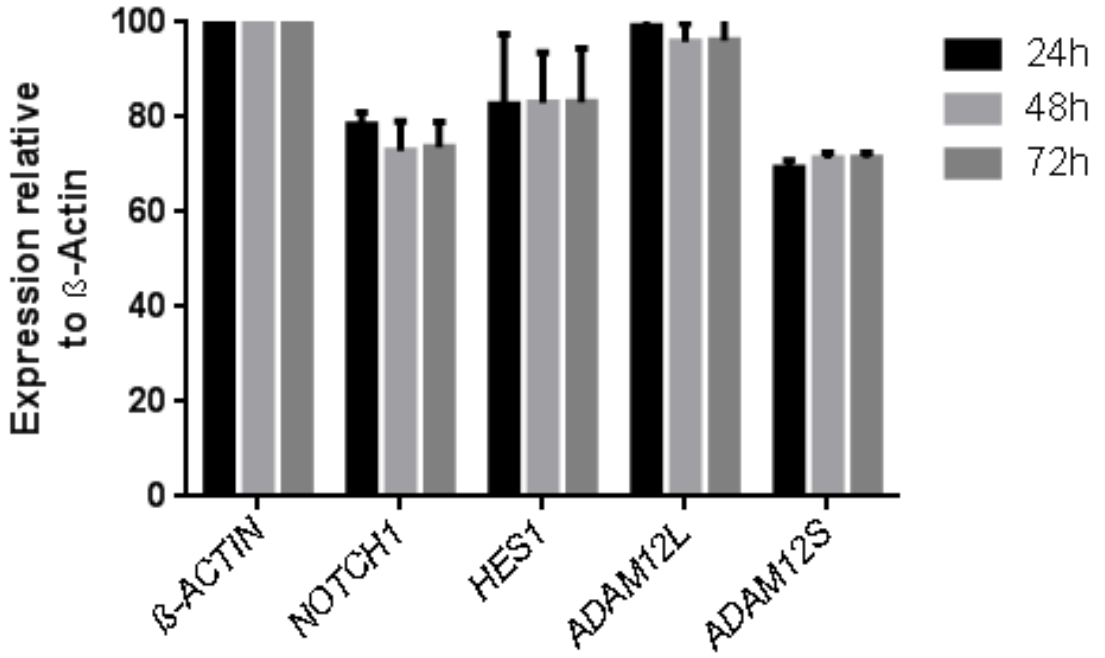

Figure 8: Expression of *NOTCH, HES1,* and *ADAM12* in U87MG GBM cell line. Quantitative RT-PCR analysis of transcription levels of *NOTCH1*, *HES1*, and *ADAM12* in U87MG GBM cell line at 24h, 48h, and 72h of culture. Results were generated from 3 independent experiments, calculated by the ΔΔCT method, and normalized to β-Actin. Each bar represents the mean ± standard error of the mean [SEM].

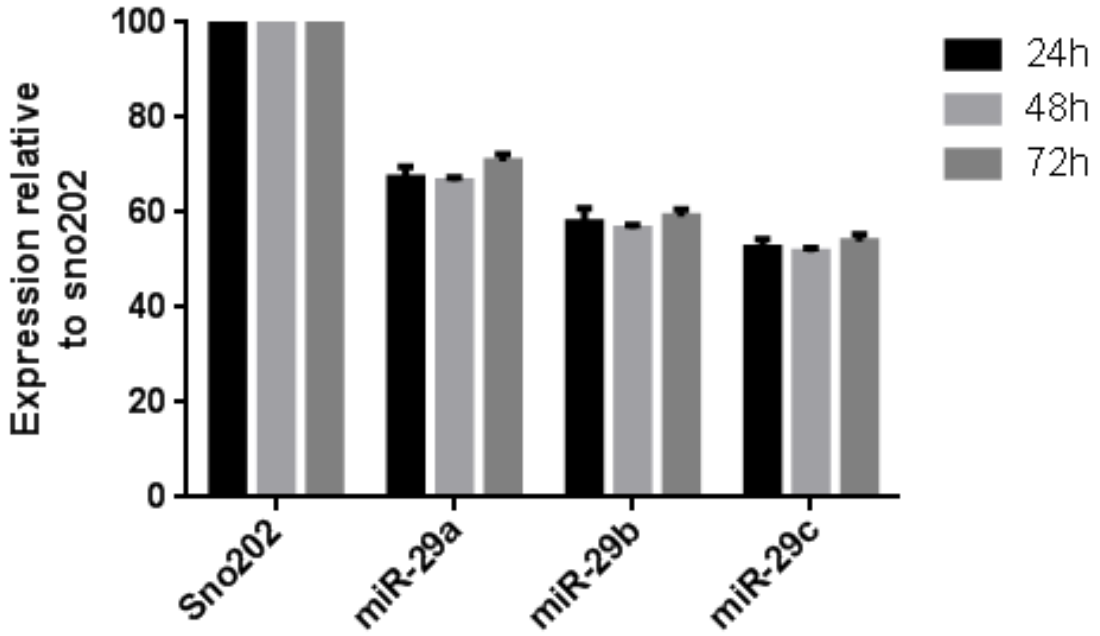

Figure 9: Expression of miR-29 family in U87MG GBM cell line. Quantitative RT-PCR analysis of transcription levels of miR-29a, miR-29b, and miR-29c in U87MG GBM cell line at 24h, 48h, and 72h of culture. Results were generated from 3 independent experiments, calculated by the ΔΔCT method, and normalized to sno202. Each bar represents the mean ± standard error of the mean [SEM].

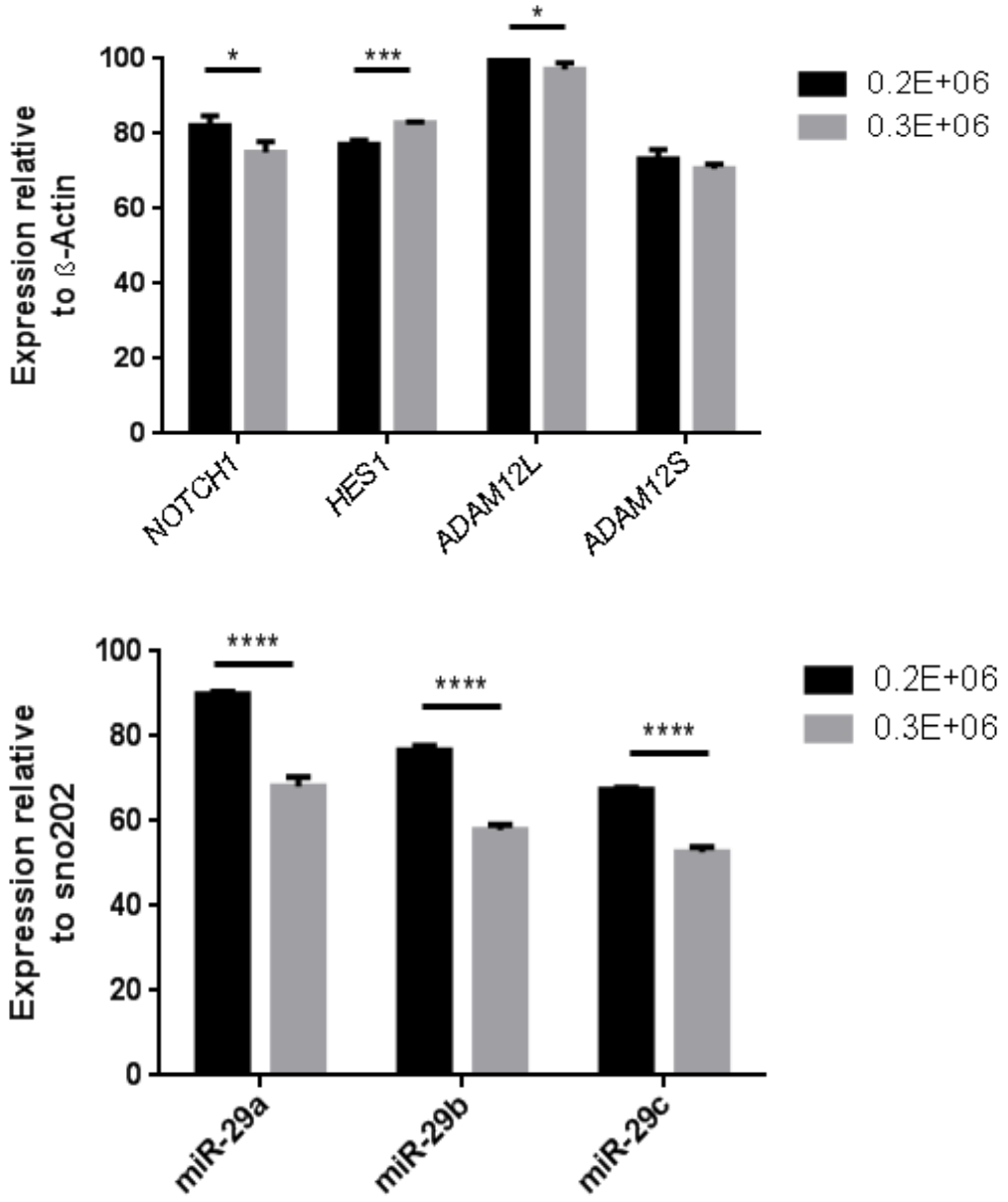

Figure 10: Expression of *NOTCH1*, *HES1*, *ADAM12*, and miR-29 family at two different cell densities of U87MG GBM cell line. Quantitative RT-PCR analysis in U87MG GBM cell line at $0.2X10^6$ and $0.3X10^6$ cells per well. Results were generated from 3 independent experiments, calculated by the ΔΔCT method. mRNA data were normalized to *β-ACTIN*, and miRNA data were normalized to sno202. Each bar represents the mean ± standard error of the mean [SEM].**P <0.05; *** P <0.001; **** P <0.0001*

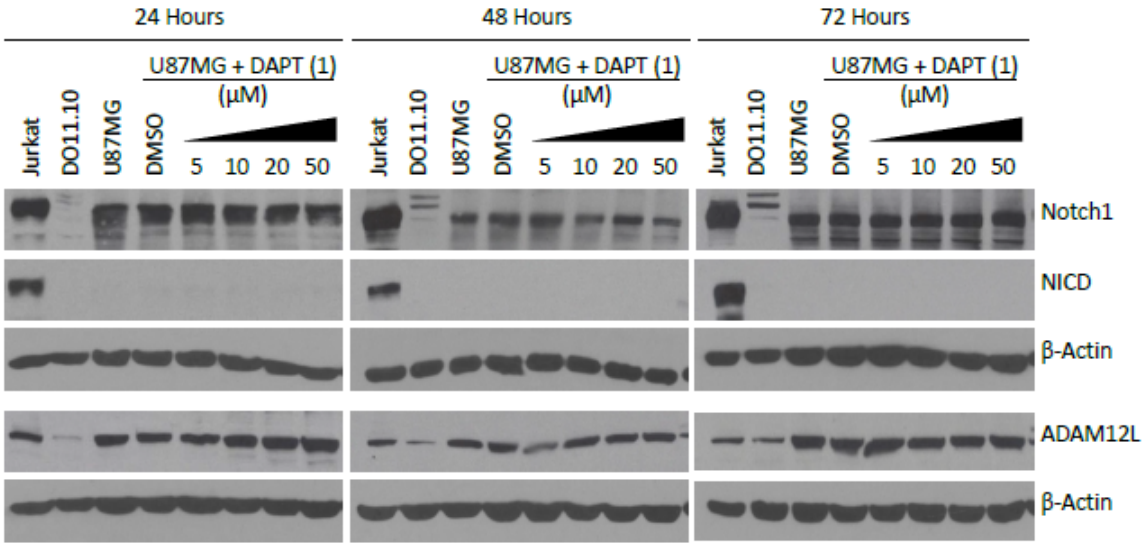

Figure 11: Effect of GSI on the expression of Notch1, cleaved intracellular Notch1, and ADAM12 in U87MG GBM cell line. Western blot analysis of protein levels of Notch1, cleaved intracellular Notch1, and ADAM12 in U87MG GBM cell line at 24h, 48h, and 72h of culture treated with 5, 10, 20, and 50μM of DAPT-GSI. Jurkat cells were used as a positive control for the expression of Notch1, cleaved intracellular Notch1, and ADAM12L and DO11.10 cells were used as a negative control for the expression of Notch1.

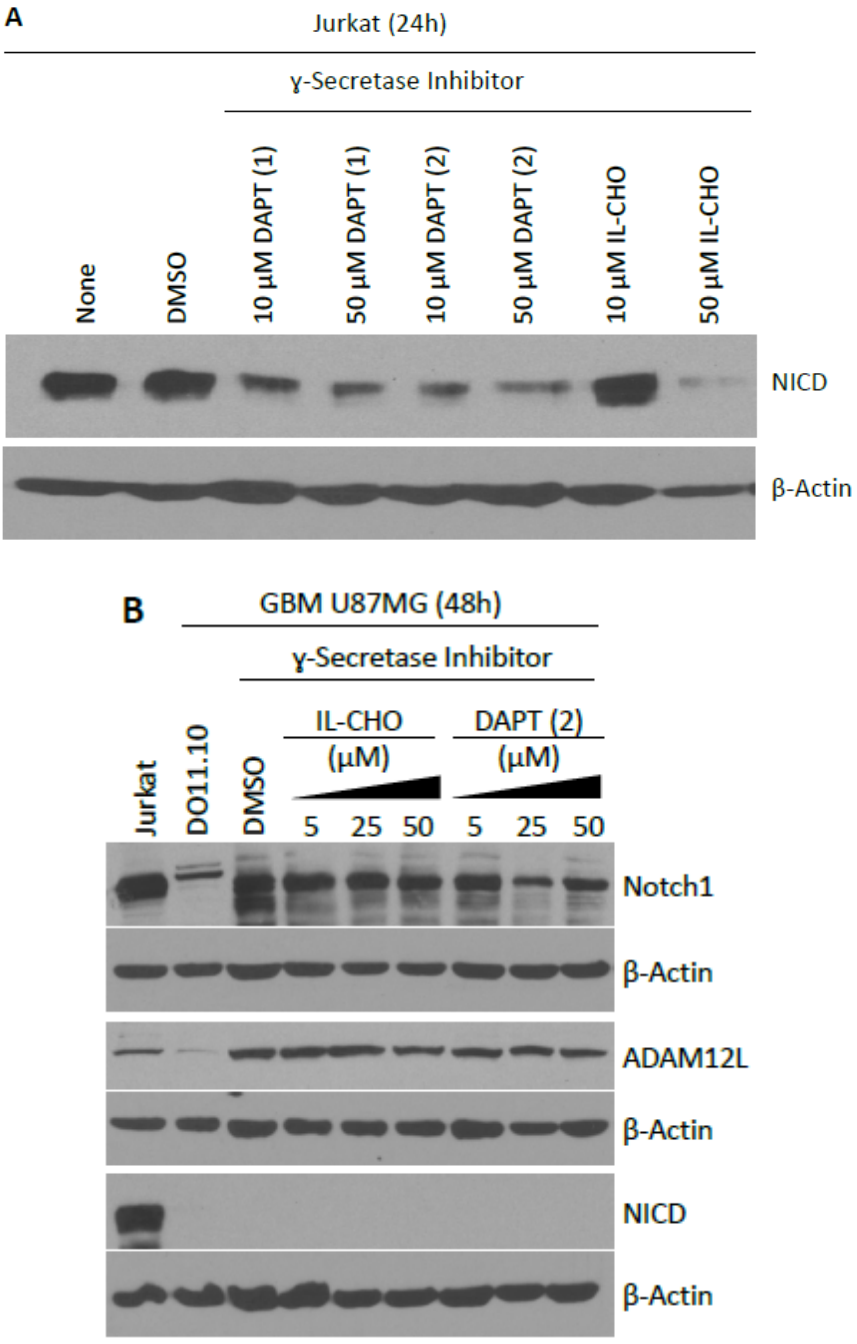

Figure 12: Effects of different GSI preparations on Notch1 activation.(A) Western blot analysis of protein levels of cleaved intracellular Notch1 in Jurkat cell line at 24h of culture treated with 10 and 50µM of two preparations of DAPT-GSI and one preparation of IL-CHO-GSI.**(B)** Western blot analysis of protein levels of Notch1, cleaved intracellular Notch1, and ADAM12 in U87MG GBM cell line at 48h of culture treated with 5, 25 and 50µM of DAPT-GSI and IL-CHO-GSI.

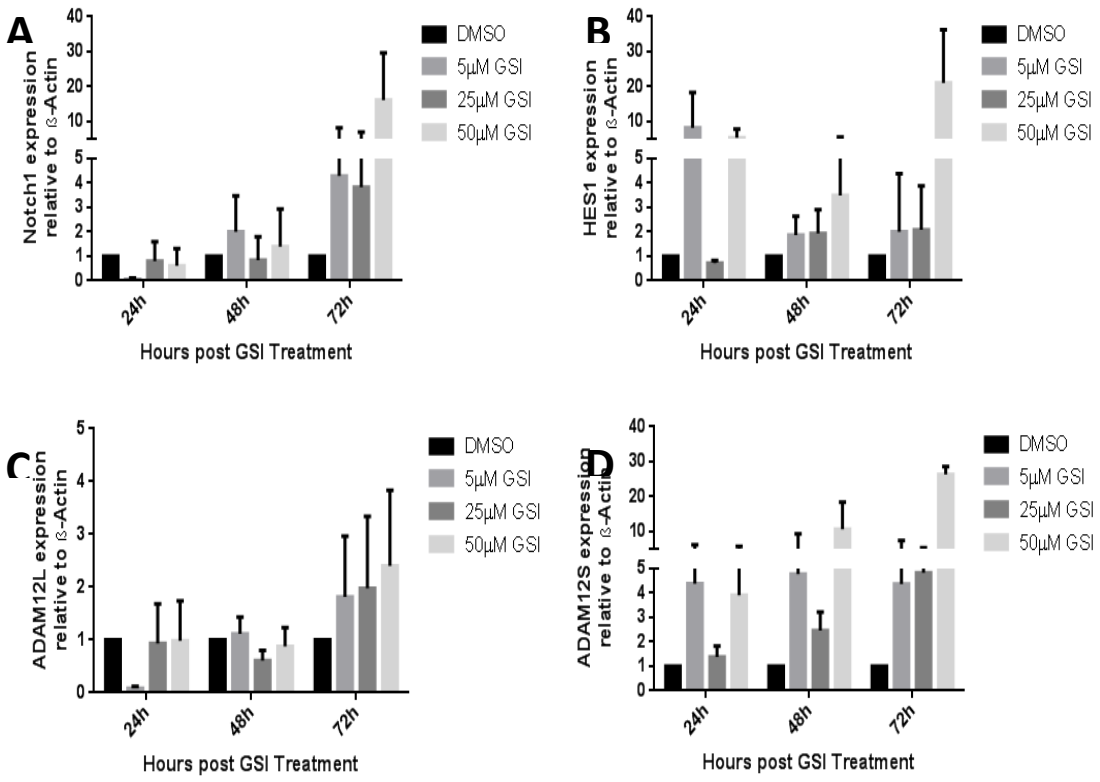

Figure 13: Effects of GSI on the expression of Notch1, cleaved intracellular Notch1, and ADAM12 in U87MG GBM cell line. Quantitative RT-PCR analysis in U87MG GBM cell line at $0.3X10^6$ cells per well and treated with 5, 25, and 50μM DAPT-GSI for 24h, 48h, and 72h. Results were generated from 3 independent experiments, calculated by the ΔΔCT method and normalized to β-Actin. Each bar represents the mean ± standard error of the mean [SEM].

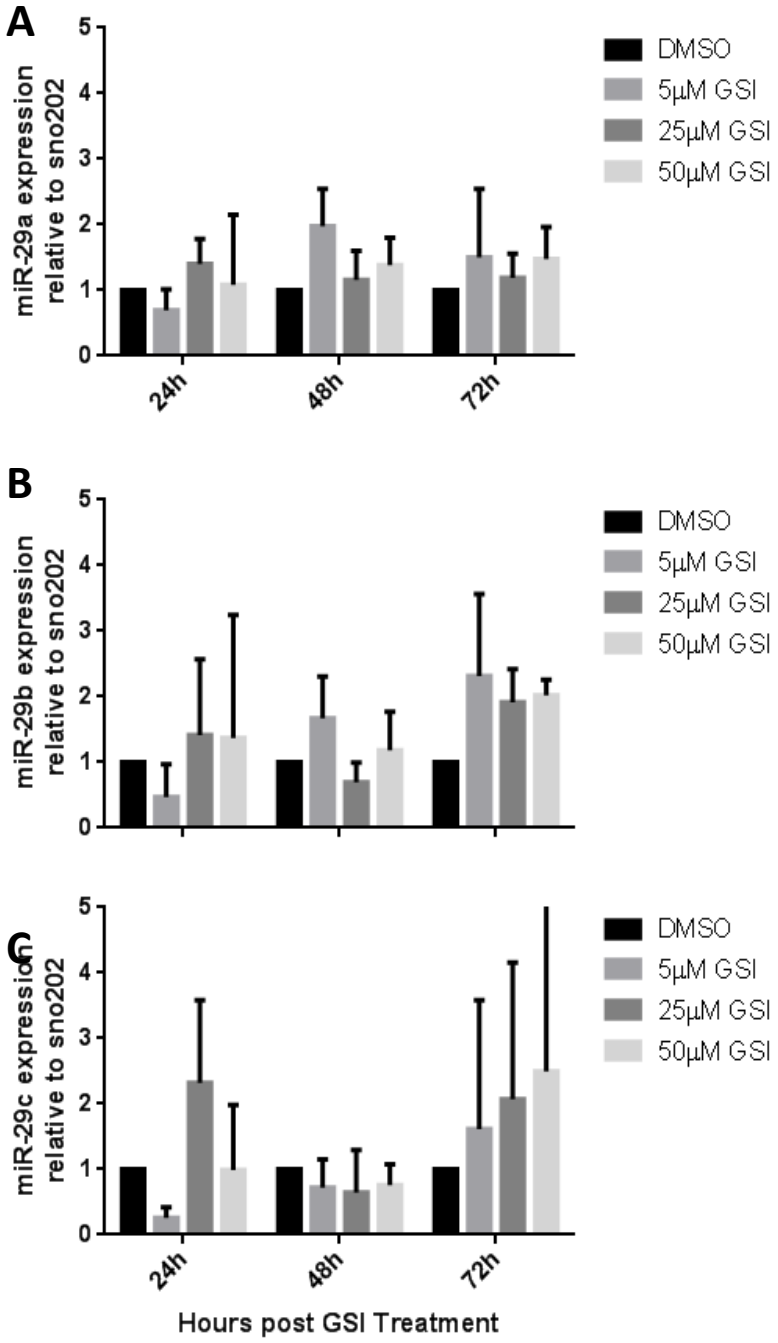

Figure 14: Effects of GSI on the expression of miR-29 in U87MG GBM cell line. Quantitative RT-PCR analysis in U87MG GBM cell line at $0.3X10^6$ cells per well and treated with 5, 25, and 50μM DAPT-GSI for 24h, 48h, and 72h. Results were generated from 3 independent experiments, calculated by the ΔΔCT method and normalized to sno202. Each bar represents the mean ± standard error of the mean [SEM].

CHAPTER 3

DISCUSSION AND CONCLUDING REMARKS

The association of Notch signaling pathway with the pathogenesis of many types of cancers has been previously demonstrated (Leong and Karsan, 2006). Here, we investigated Notch1 regulation of ADAM12 in the U87MG Glioblastoma cell line, to aid in our understanding of the pathogenesis of GBM, the most malignant type of brain cancer. We demonstrated elevated levels of Notch1, HES1, c-Myc, and ADAM12 expression levels in U87MG GBM cell line, indicating that the Notch signaling pathway is activated in GBM and correlates with the expression level of ADAM12 protein expression. We further confirmed the elevated expression of *NOTCH1*, *HES1*, and *ADAM12* in GBM cell line at the transcriptional level using quantitative RT-PCR.

To examine the downstream components of the Notch signaling pathway, we examined the expression levels of cleaved intracellular Notch1, HES1, and ADAM12. While our results demonstrated that Notch1 had been cleaved, as detected by the cleaved transmembrane component, we were unable to detect cleaved intracellular component, suggesting a dysfunction in the cleavage at the S2 cleavage site limiting the exposure of S3 cleavage site and resulting in a cleaved transmembrane with absence of the intracellular domain. Alternatively, there might be a different species of intracellular cleaved Notch that might have resulted from cleavage at a site different from the originally accepted V1744 site (Tagami et al., 2008), resulting in failure of our antibody, which only detects Notch1 cleaved at V1744, to detect the intracellular cleaved form of Notch1. Transcriptional levels of these genes on the other hand were elevated with

treatment of GSI, an inhibitor of intracellular Notch cleavage, indicating an upregulation of gene expression with post-transcriptional suppression of translation. The difference between the transcriptional and protein levels of the examined genes requires additional exploration of the mode of regulation in GBM. Furthermore, multiple types of GSI investigational drugs exist that potentially target different isoforms of ɣ-secretase present in GBM cell lines and different from those present in our Jurkat cells positive control (Kopan and Ilagan, 2009). Alternative experimental approaches can be potentially used to suppress Notch signaling pathway, such as siRNA against Notch mRNA or the expression of dominant negative form of Notch coactivator mastermind-like 1 (Chen et al., 2010).

Similarly, transcriptional levels of miR-29 members were elevated in U87MG GBM cell line, indicating their involvement in GBM pathogenesis. It is possible that the elevated miR-29 levels results in posttranscriptional suppression of ADAM12 translation, hence explaining the elevated *ADAM12* transcripts with no demonstrable elevation of ADAM12 protein levels upon GSI treatment. A similar mechanism might be involved in the regulation of Notch1 translation.

The involvement of Notch signaling pathway in brain development and cancer is highly context-dependent (Lardelli et al., 1994). Previous reports have demonstrated the differential expression of various Notch signaling components in GBM (Chen et al., 2010). Although all cell lines used were derived from GBM patients, the role of Notch signaling differed according to GBM subtype. In contrast to our results, Notch2 was the predominant Notch type involved in the majority of GBM subtypes, including U87 cell line. Notch1 expression was predominant in only 2 of the 5 cell line examined. While the

authors demonstrated the biological effect of Notch signaling on tumor growth and neurosphere formation, they similarly failed to present the expression of cleaved intracellular Notch domain. It is then likely that Notch exerts its function in GBM through a different pathway that does not induce the expression of cleaved intracellular Notch domain, or that we are temporally unable to detect that component in our culture conditions due to a short half-life of the cleaved protein.

Other studies have indicated that Notch signaling increases neurosphere formation (Fan et al., 2010; Gilbert et al., 2010; Jeon et al., 2008), the cancer stem cell component of GBM, indicating the need to test our parameters in neurosphere culturing conditions, which include serum depletion of culture media and longer culturing durations of up to 10 days.

In all, our study supports the role of Notch signaling pathway in GBM pathogenesis and support further investigation in the role of ADAM12 and miR-29 in the pathogenesis of GBM.

CHAPTER 4

MATERIALS AND METHODS

4.1. Cell Culture

U87MG and Hela cells were cultured in EMEM. DO11.10 cells were cultured in RDG complete media composed of 45% RPMI 1640 and 45% DMEM. Jurkat cells were cultured in RPMI 1640 supplemented with 1% L-glutamate, 1% Na pyrovate, and 2-mercaptoethanol. All culture media were supplemented with 10% FBS and 1% penicillin-streptomycin (Lonza, Switzerland) and incubated at 37°C, 5% CO_2. Hela, Jurkat, and DO11.10 cells were cultured to 80% confluence for protein extraction. U87MG cells were plated as described in "Compound Treatment" section below.

4.2. Viability Analysis

U87MG cells were cultured at $0.2X10^6$, $0.5X10^6$, and $1X10^6$ for 24h and 48h. At the end of each time point, 0.5 ml of a cell suspension was placed in a screw cap test tube and mixed thoroughly with 0.1 ml of 0.4% Trypan Blue Stain. Cells were allowed to stand 5 min at room temperature. Dead cells, stained blue, were counted in a hemocytometer under a microscope. Percentage of viable cells was calculated by dividing the total number of unstained cells over the total number of cells.

4.3. Compound treatment

U87MG cells were plated at different cell numbers, as discussed in the results sections, in 2ml growth medium per well in 6-well plate. Twenty four hours later, cells were treated with 2, 5, 10, 20, 25, and 50 μM (in DMSO) of GSI inhibitors cbz-I-L-CHO (zIL-CHO) or N-[N-(3, 5-difluorophenacetyl)-l-alanyl]-5-phenylglycine t-butyl ester (DAPT)

(Sigma-Aldrich) for 24, 48, and 72 hours. Additional wells were either left untreated or treated with comparable volumes of DMSO (Sigma-Aldrich).

4.4. Protein Extraction

Whole cell lysates were prepared by lysing cells using RIPA buffer (50mM Tris, pH8.0, 150mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors). For subcellular fractionation, total cellular protein was separated into cytoplasmic and nuclear fractions using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific), according to manufacturer's directions. Protein concentrations were measured using BCA Assay kit according to manufacturer's directions (Thermo Scientific).

4.5. Antibodies and Western Blot Analysis

Thirty to fifty micrograms of protein were separated by electrophoresis in 8% SDS-polyacrylamide gels. Proteins were electrotransferred to polyvinylidenedifluoride membranes (PVDF) (Millipore, Bedford, MA), while either immersed in 1X transfer buffer (wet transfer) or without transfer buffer (semi-dry transfer), and probed with the indicated primary antibodies as shown in table 2. Horseradish peroxidase-conjugated donkey anti-rabbit and sheep anti-mouse secondary antibodies (GE Health, Piscataway, NJ) were used at a dilution of 1:2500, and bands were detected with enhanced chemiluminescence (ECL, Thermo Scientific). Loading was normalized with β-Actin (Sigma-Aldrich) for total and cytoplasmic proteins, or Histone Deacetylase 1 (HDAC1) for nuclear proteins. Solution for blocking membranes and for primary and secondary antibody dilutions (BLOTTO) was made from 1X PBS containing 0.1% Tween-20 supplemented with 5% (wt/vol) dry fat-free milk powder (Carnation Nestle).

Table 2: Primary Antibody List

Antigen	Species	Clone	Dilution	Source
Notch1	Rabbit	sc-6014R	1:200	Santa Cruz
Notch1	Mouse	mN1A	1:1000	eBioscience
Cleaved Notch1	Rabbit	Polyclonal	1:1000	Cell Signaling
ADAM12	Rabbit	Polyclonal	1:1500	Proteintech
Hes1	Rabbit	Polyclonal	1:1000	Santa Cruz
c-Myc	Rabbit	Polyclonal	1:500	Santa Cruz
β-Actin	Mouse	AC-40	1:3000	Sigma-Aldrich
HDAC1	Rabbit	Polyclonal	1:1000	Cell Signaling

4.6. Quantitative Real-time Reverse Transcription PCR (qRT-PCR)

Total RNA was isolated using mirVana^{TM} miRNA isolation kit (Ambion) according to the manufacturer's directions, and RNA concentration was measured by nanodrop analysis. cDNA was generated from 1µg of total RNA (denatured for 5 minutes at 65°C) in 1.2µl of dNTPs (Invitrogen), 2µl of M-MuLV reverse transcriptase buffer (New England Biolabs), 1µl of oligo-DT (Invitrogen), 1µl RNase inhibitor (Promega) and 1µl of 200.000 U/ml M-MuLV reverse transcriptase (New England Biolabs) performed at 42°C for 45 minutes followed by 65°C for 10 minutes using Mater Cycler Pro (Eppendorf). cDNA for microRNAs was synthesized from 0.1µg of RNA using 1µl of 2pM of each stem loop primer. Resulting cDNA was diluted at 1:5 for total RNA gene-specific amplification, undiluted for microRNA, and 1:100 for housekeeping gene (*β-ACTIN*) controls and reaction was setup using 1µl cDNA, 10µl of SYBR green mix (Takara), 0.4µl of 10µM forward primer, 0.4µl of 10µM of reverse primer, and 8.2µl of water. Reactions were performed in duplicates per cDNA sample using MxPRO 3000 (Stratagene) (95°C 5 min, 40 cycles [95°C X 25 sec, 62°C X 25 sec, 95°C X 1 min, 62°C X 1 min, 95°C X 30 sec). The relative change of transcript amount in each sample was determined by normalizing with the *β-ACTIN* mRNA (or *Sno202* for microRNA) expression levels using the ΔΔCT method. Primer sequences are listed in Table 3.

Table 3: Primers for RT-PCR and qRT-PCR

Symbol	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
NOTCH1	GAGGCGTGGCAGACTATGC	CTTGTACTCCGTCAGCGTGA
ADAM12L	GTTTGGCTTTGGAGGAAGCACAG	TGCAGGCAGAGGCTTCTGAGG
ADAM12S	CAAGGAGGCCGGATTCTGTG	TCAGATGAGTGTCAGTGA
HES1	GAGAGGCGGCTAAGGTGTTTG	CTGGTGTAGACGGGGATGAC
β-ACTIN	GTTGTCGACGAGCG	GCACAGAGCCTGGCCTT
miR-29a	GTGCCGTACTAGCACCATCTG	GCGACTTAGCTAACCGATTTC
miR-29b	GGCGCGTAGCACCATTTGAAA	GCGACACTAACCAACACTGAT
miR-29c	GTGCCGTCCTAGCACCATTTG	GCGACTTAGCTAACCGATTTC
Sno202	GCTGTACTGACTTGATGAAAG	CATCAGATGGAAAAGGCTTCA
miR-29b stem loop	GGTTAGACACAAGCGACACTAACCAACACT	
miR-29ac stem loop	GCTAAGACCATCATGCGACTTAGCTAACCG	

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